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Neurotrophic activity of jiadifenolide on neuronal precursor cells derived from human induced pluripotent stem cells

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ABSTRACT

Although jiadifenolide has been reported to neurotrophin-like activity in primary cultured rat cortical neurons, it is unknown on that of activity in human neurons. Thus, we aimed to assess neurotrophin-like activity by jiadifenolide in human neuronal cells. We analyzed neuronal precursor cells derived from human induced pluripotent stem cells for microtuble-associated-protein-2 expression by immunofluorescence and western blot, following jiadifenolide treatment. Jiadifenolide promoted dendrite outgrowth, facilitated growth, and prevented death in neuronal cells derived from human induced pluripotent stem cells. Interestingly, jiadifenolide also increased postsynaptic density-95 protein expression suggesting that jiadifenolide promotes neuronal maturation and post-synaptic formation. We demonstrate for the first time that jiadifenolide exhibits neurotrophic effects on human neuronal precursor cells.

1. Introduction

Neurotrophic factors (neurotrophins) such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) have been reported to prevent neuronal degeneration, promote neurite regeneration and neuronal survival, and enhance synaptic plasticity [1,2]. Thus, neurotrophins have been investigated for therapeutic application in neurological diseases including Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis (ALS) that show neurodegenerative, behavioral, and psychiatric symptoms [3,4]. However, although neurotrophins act largely within the brain, these proteins have high molecular weights, limiting serum-stability and blood-brain-barrier passage [5–7]. Moreover, clinical trials of NGF and BDNF have failed [8]. Thus, novel small-molecule neurotrophin-like compounds are needed, which lack such pharmacological disadvantages.

Jiadifenolide (Fig. 1), a seco-prezizaane-type sesquiterpenoid, was isolated from the pericarps of *Illicium jiadifengpi* collected in Southwestern China [9], and was chemically synthesized [10]. Jiadifenolide has very unique chemical structure (Fig. 1) [9, 10]. Jiadifenolide and derivatives have been reported to promote neurite outgrowth in primary cultured rat cortical neurons [9] and enhance NGF activity, thereby facilitating differentiation of PC12 cells (a rat adrenal pheochromocytoma cell line) [11]. Thus, previous studies suggest these compounds have neurotrophin-like effects in rat neurons. However, owing to species differences, there have been limitations in accurately predicting the clinical activities and adverse effects of jiadifenolide in human neurons. Therefore, prior to conducting clinical trials, an investigation of the neurotrophic effects of jiadifenolide on human neurons is necessary.

2. Materials and methods

2.1. Preparation of jiadifenolide

Jiadifenolide (Fig. 1) as chemically synthesized from neomajucin isolated from the pericarps of *Illicium jiadifengpi*, and purified as previously reported [9]. We dissolved synthetic jiadifenolide in dimethyl sulfoxide (DMSO) at a concentration of 10 mM for stock solutions.

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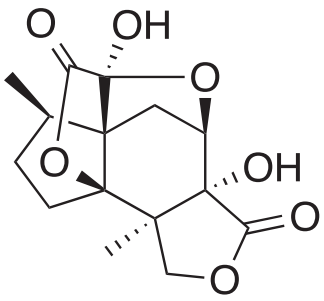

jiadifenolide

Fig. 1. Chemical structure of jiadifenolide.

2.2. Culture of human induced pluripotent stem cells (hiPSCs)

Human induced pluripotent stem cells (hiPSCs) (line 201B7; Cell No. HPS0063) [12], were purchased from a cell bank (Riken Bioresource Center, Ibaraki, Japan). Cells were maintained on a mitomycin-C (Kyowa Hakko Kirin, Tokyo, Japan)-treated SNL 76/7 feeder-cell (DS Pharma Biomedical, Osaka, Japan) layer at 37 °C and 5% CO_2. Cells were grown in a primate embryonic stem cell medium (ReproCELL, Kanagawa, Japan), supplemented with 4 ng/mL basic fibroblast growth factor (Wako, Osaka, Japan) and 50 U/mL penicillin with 50 μg/mL streptomycin (P/S; Life Technologies, CA, USA).

2.3. Induction of neuronal precursor cell differentiation

Differentiation of neuronal precursor cells from hiPSCs was induced by serum-free culture of embryoid body-like aggregates, quick (SFEBq) method [13,14] (Fig. 2, stages I and II). Briefly, hiPSCs cultured on a mitomycin C-treated SNL feeder-cell layer were treated with 10 μM Y-27632, Rho-associated coiled-coil forming kinase/Rho-associated kinase inhibitor, (Wako, Osaka, Japan), and were dissociated into single cells using TrypLE™ Select solution (Life Technologies) after removing feeder cells using CTK solution (2.5% trypsin [Life Technologies], 1 mg/mL collagenase IV [Life Technologies], 0.1 M $CaCl_2$ and 20% knockout serum replacement [KSR; Life Technologies] in H_2O). The hiPSCs were aggregated in a low-binding, U-bottomed, 96-well plate at 37 °C and 5% CO_2 and 5% DFK medium (5% KSR [Life Technologies], 100 μM modified Eagle medium, nonessential amino acids solution [Life Technologies], 2 mM L-glutamine, 110 μM 2-mercaptoethanol [Life Technologies], and P/S [Life Technologies] in glucose-Dulbecco's modified Eagle medium (DMEM)/F12 medium [Life Technologies], supplemented with 10 μM Y-27632, 2 μM dorsomorphin (Calbiochem, CA, USA) and 10 μM SB431542, transforming growth factor β1 superfamily activin receptor-like kinase inhibitor, (Stemgent, MA, USA) for embryoid-body (EB) formation and neuronal induction (Fig. 2, stage I). Half of media in wells was changed with 5% DFK medium at either 3 or 4 days of incubation. After 10–12 days of incubation, EBs were transferred onto a Matrigel matrix (Corning, MA, USA)-coated 6-well plate and cultured for 16 days at 37 °C and 5% CO_2 with 5% DFK medium supplemented with 1% N2 supplement (Life Technologies), 2 μM dorsomorphin, and 10 μM SB431542. Neuronal precursor cells were removed from EB cores [14] (Fig. 2, stage II).

2.4. Maturation of neuronal cells

The procedure used to induce neuronal maturation was performed as indicated in Fig. 2, stage III. Neuronal precursor cells removed from EB cores were dissociated into single cells with an accutase (Life Technologies), and were then cultured on both 0.01% poly-L-ornithine (Sigma, MO, USA) and 3.3 μg/mL laminin (Sigma)-coated 24-well plates at 37 °C and 5% CO_2, with neurobasal medium (Life Technologies), supplemented with 2% B-27 supplement (Life Technologies) and P/S. Depending on treatment conditions, media also contained one of the following: (i) 0.25% DMSO (as a negative control); (ii) neurotrophins (as a positive control; 10 ng/mL recombinant human BDNF [Wako], 10 ng/mL recombinant human glial-cell derived neurotrophic factor [GDNF; Wako], and 10 ng/mL recombinant human NT-3 [Wako] [14]); or (iii) 1, 5, 10, or 25 μM jiadifenolide (Fig. 2, stage III). Media in wells was changed at either 3 or 4 days of incubation.

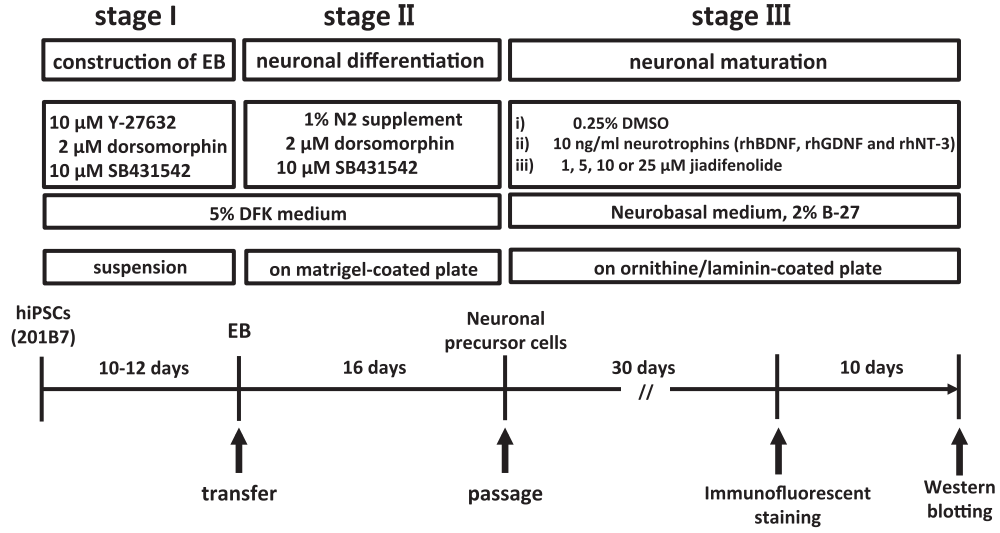

Fig. 2. Experimental procedure for neuronal cell differentiation and maturation from hiPSCs. To evaluate the neurotrophic effects of jiadifenolide on human neurons, Jiadifenolide was added at stage III. Immunofluorescent staining and western blots were performed on neuronal precursor cells derived from hiPSCs treated with (i), (ii), or (iii), at 30 or 40 days post-incubation, respectively. Additional details are described in Materials and Methods.

2.5. Immunofluorescent staining

At 30 days of incubation (Fig. 2, stage III), mature cells were fixed with Cytofix™ fixation buffer (Becton Dickinson [BD], NJ, USA) for 10 min at room temperature (R/T), and permeabilized with BD Phosflow™ Perm Buffer III (BD) for 5 min at R/T. After washing cells with Perm/Wash™ Buffer solution (BD), the cells were blocked using Pharmingen™ Stain Buffer (with fetal bovine serum) (BD). For neuronal dendrite immunofluorescent staining, we used primary mouse anti-microtubule-associated protein 2 (MAP2; a neurite marker) monoclonal antibodies (HM-2, Abcam, Cambridge, UK), and Alexa fluor 488-conjugated goat anti-mouse IgG secondary antibodies (Life Technologies). Cell nuclei were then stained using diamidino-2-phenylindole (DAPI; Life Technologies). Wells were photographed under a fluorescence microscope (BIOREVO BZ-9000, Keyence, Osaka, Japan). Using the IN Cell Investigator image-analysis software (GE Healthcare, Bucks, UK), the total neurite area (mm^2), total neurite length (mm), and neuronal cell numbers were measured as based on MAP2-positive area, length, or DAPI-positve cell numbers, respectively.

2.6. Western blotting

At 40 days of incubation (Fig. 2, stage III), cells were lysed with a sodium dodecyl sulfate buffer (125 mM Tris–HCl [pH 6.8], 5% sodium dodecyl sulfate, 25% glycerol, 0.1% bromophenol blue, and 10% β-mercaptoethanol) and boiled for 5 min. Cell lysates were then loaded onto a 6–10% polyacrylamide gel. Resulting proteins were transferred to a polyvinylidene fluoride microporous membrane (Millipore, MA, USA). Mouse anti-MAP2 monoclonal (HM-2, Abcam) or rabbit anti-postsynaptic density-95 (PSD95; a post-synaptic marker) polyclonal (ab18258, Abcam) primary antibodies were used to detect MAP2 or PSD95 proteins, respectively. Rabbit anti-β–actin antibodies (13E5; Cell Signaling, MA, USA) were used as an internal control. Horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (SouthernBiotech, AL, USA), or goat anti-rabbit IgG antibodies (KPL, MD, USA) were used as secondary antibodies. Blots were developed using Western Lightning ECL Pro (PerkinElmer, MA, USA). Protein-band intensities were measured using ImageJ software. MAP2 and PSD95 protein levels were normalized to that of β-actin.

2.7. Statistical analysis

All results are expressed as means ± standard error of the mean (SEM). Differences were analyzed for statistical significance using one-way analysis of variance (ANOVA), which facilitates comparison among more than three groups. Significance levels were determined at $p < 0.05$.

3. Results

3.1. Jiadifenolide promoted neuronal dendritic outgrowth, and increased human neuronal cell numbers

It has been reported that neurotrophins, such as NGF, BDNF, NT-3, and GDNF, promote neurite regeneration, neuron survival, and synapse maturation, including synaptic construction and plasticity [1,2,15]. To investigate whether jiadifenolide exhibits neurotrophic activity in human neurons, we therefore used neuronal precursor cells induced from hiPSCs using SFEBq method (Fig. 2).

To evaluate the neurite outgrowth, we first performed immunofluorescent staining for MAP2, neuronal dendritic marker, at 30 days of incubation, as shown in Fig. 2, stage III. Compared with 0.25% DMSO (a negative control), neurotrophins, (a mixture of BDNF, GDNF and NT-3; a positive control), and 1 or 10 μM jiadifenolide promoted neurite extension (Fig. 3A), and significantly increased total neurite area and length (Fig. 3B and C) in iPSC-derived neuronal cells. These results demonstrate that jiadifenolide promotes dendrite outgrowth in human neurons. Additionally, treatment with both neurotrophins and 1 μM jiadifenolide significantly increased the number of neuronal cells (DAPI-positive cells) (Fig. 3D), suggesting that jiadifenolide promoted the growth, or prevented the death, of human neuronal cells.

Neurotrophic effects were achieved at similar jiadifenolide concentrations for human neuronal cells (Fig. 3) as those for primary cultured rat cortical neurons [9]. These data demonstrated that, similar to rat neurons, jiadifenolide exhibits neurotrophic effects in human neuronal cells.

3.2. Jiadifenolide promoted synaptic maturation in human neuronal cells

To evaluate whether jiadifenolide promotes synaptic maturation in human neuronal cells, we analyzed the protein expression of MAP2 and PSD95 (a post-synaptic marker) using western blotting at 40 days of incubation (Fig. 2, stage III). Compared with 0.25% DMSO, neurotrophins and jiadifenolide (1 and 5 μM) significantly increased MAP2 protein expression in neuronal cells derived from hiPSCs (Fig. 4A and B). Interestingly, we also found that 1 and 5 μM jiadifenolide significantly increased PSD95 protein expression (Fig. 4A and C). It has been reported that the increase of PSD95 expression in neurons promotes the maturation of excitatory synapses, such as those in glutamatergic neurons [16–18]. These data suggest that jiadifenolide promotes synaptic maturation in human neurons.

4. Discussion

AD is characterized by neuronal and synaptic degeneration, apoptosis, neuroinflammation, and autophagy, which are induced by accumulation of abnormally folded amyloid-β (Aβ) and tau proteins [19]. These neurodegenerative events can subsequently lead to behavioral and psychiatric disorders including depression, agitation, and apathy [20]. Among these symptoms, depression is frequency observed in patients with AD, with a prevalence of between 20 and 50% [20]. Shankar et al. reported that Aβ protein dimers derived from the brains of human patients with Alzheimer's disease impaired synaptic plasticity, reduced dendritic spine density, and disrupted memories of learned behaviors in rats [21]. In transgenic AD mice, loss of the pre-synaptic protein synaptophysin, the post-synaptic protein PSD95, and the glutamate receptor subunit GluR1, have also been observed [22–26]. Reduction of neurogenesis and synaptophysin and the loss of dendritic spines have also been observed in rat depression models [27,28]. We demonstrated that jiadifenolide promoted dendritic outgrowth and overall cell growth (or prevented cell death), and increased MAP2 and PSD95 protein expression in human neurons (Figs. 3 and 4). These results suggest that jiadifenolide may improve characteristic symptoms, such as depression, in patients with AD. We showed that, among concentrations of 1–25 μM, 1 μM jiadifenolide had the greatest efficacy on the neurons in our study (Figs. 3 and 4). This lower-dose efficacy was similar to that observed in primary cultured rat cortical neurons [9]. In experiments using ethanol-treated rats, low doses of ethanol improved memory and increased NMDA-receptor GluN1 subunit expression, whereas high doses of ethanol reduced neurogenesis, diminished NMDA-receptor GluN2B subunit expression, and impaired visual memory [29]. In addition, low ethanol increased the expression of tropomyosin receptor kinase (Trk) B receptor protein in CA3 region of rat

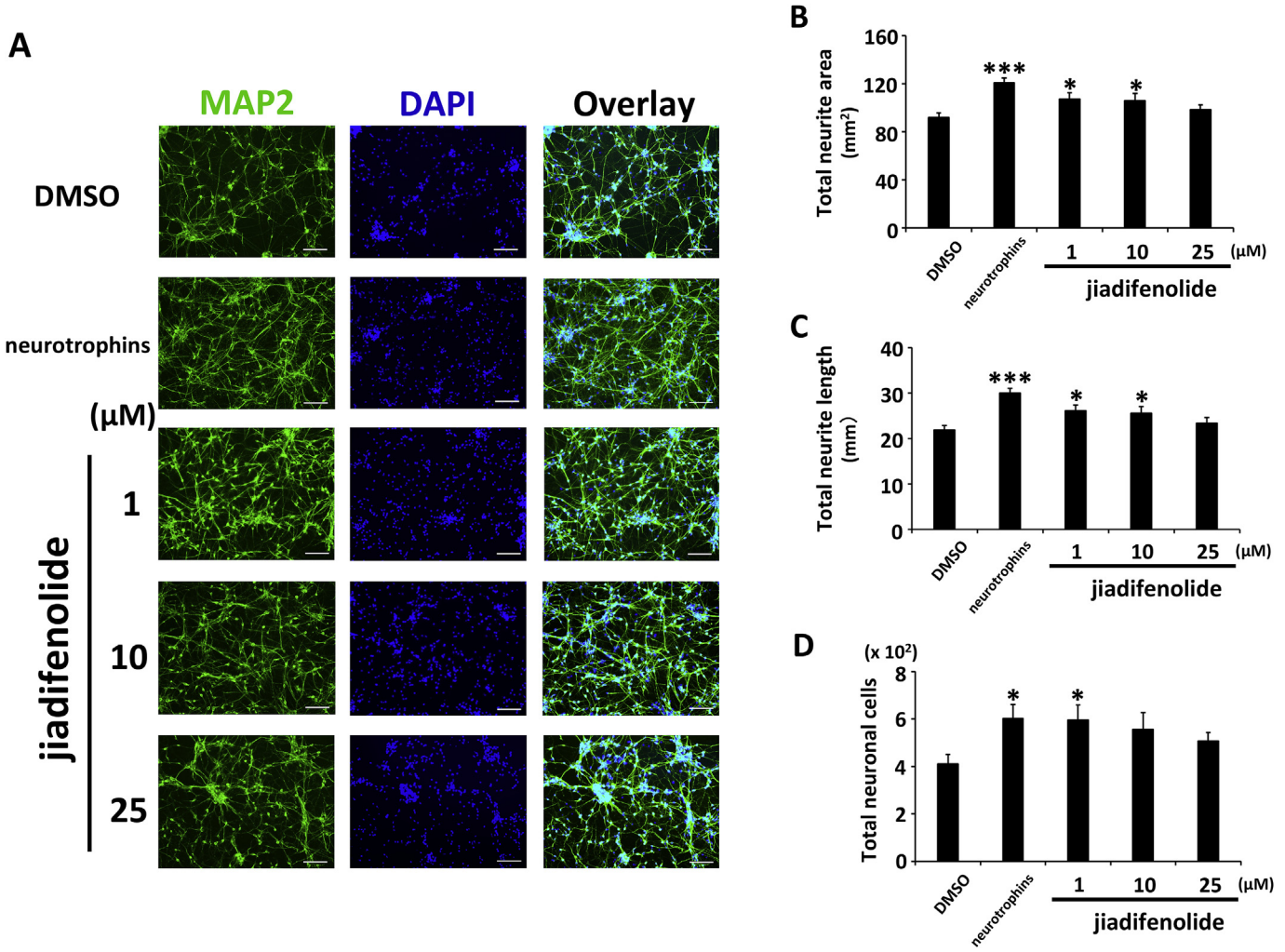

Fig. 3. Immunofluorescent MAP2 staining of human neuronal cells treated with jiadifenolide. At 30 days of incubation (Fig. 2, stage III), the cells were fixed and permeabilized. To visualize neuronal dendrites, we performed immunofluorescent staining for MAP2, a neurite marker, and DAPI, a nucleus-staining compound. Each well was treated with (i) 0.25% DMSO, (ii) neurotrophins or 1–25 μM jiadifenolide, respectively. Cells were subsequently photographed under a fluorescence microscope (A). The white scale bar in each image represented 100 μm. Images in (A) (n = 6 each) were analyzed using IN Cell Investigator image-analysis software. Total neurite area (mm^2) (B), total neurite length (mm) (C), and the number of neuronal cells (DAPI-positive cells) (D) were measured. Data are presented as means ± SEM of three independent experiments. $^*p < 0.05$ and $^{***}p < 0.001$, compared with DMSO treatment, respectively.

brain, and the mRNA levels of BDNF and TrkB were increased in the dorsal hippocampus of ethanol-fed rats [29,30]. Thus, these results suggest that low ethanol would improve the memory by increasing NMDA-receptor GluN1 subunit expression and enhancing BDNF-TrkB neurotrophic pathway. In a similar manner, moderate jiadifenolide doses may lead to neurotrophic effects, both in human and rat neurons.

Although we showed that jiadifenolide exhibits neurotrophic activity in human neuronal precursor cells, the underlying molecular mechanisms remain unclear. Neurotrophins, such as NGF, BDNF and NT-3, act via two receptors: the p75 neurotrophin receptor ($p75^{NTR}$) and the Trk receptor. $p75^{NTR}$ binds to all neurotrophins extracellularly; this signal is then transduced intracellularly via the phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and nuclear factor-κB (NF-κB) pathways [1,2]. Signaling via $p75^{NTR}$ induces cell survival, cell death, regulation of proliferation, and inhibition of neurite outgrowth [1,2]. Trk receptors consist of the TrkA, TrkB and TrkC subtypes, which have intracellular-tyrosine-kinase and extracellular-neurotrophin-binding domains. Each receptor subtype binds to a different neurotrophin: NGF binds with TrkA, and BDNF and NT3 bind with TrkB and TrkC, respectively [1,2]. Each neurotrophin-Trk receptor interaction induces phosphorylation of intracellular-domain tyrosine residues via autophosphorylation [1,2]. Trk-phosphorylation signaling is then transmitted to the MAPK, PI3K, or phospholipase Cγ1 (PLCγ) pathways, which ultimately promotes neurite outgrowth, cell survival, and differentiation [1,2]. Additionally, BDNF signaling increases PSD95 protein expression in synapses and dendritic spines [31,32], and the MAPK, PI3K, and PLCγ pathways mediated via TrkB regulate post-synaptic localization of PSD95 [33]. Taken together, these results suggest that TrkB signaling pathways may be implicated in the neurotrophic effects of jiadifenolide on human neurons.

Cells differentiated from hiPSCs, such as neurons and hepatocytes, have been used as a new tool to predict the clinical safety and efficacy of drug candidates [34]. Pre-clinical experiments using cells derived from hiPSCs have the advantages of overcoming species differences, improving the replacement, refinement, and reduction of animal experiments, and reducing drug-development costs. Pardridge has suggested in a review that small-molecule

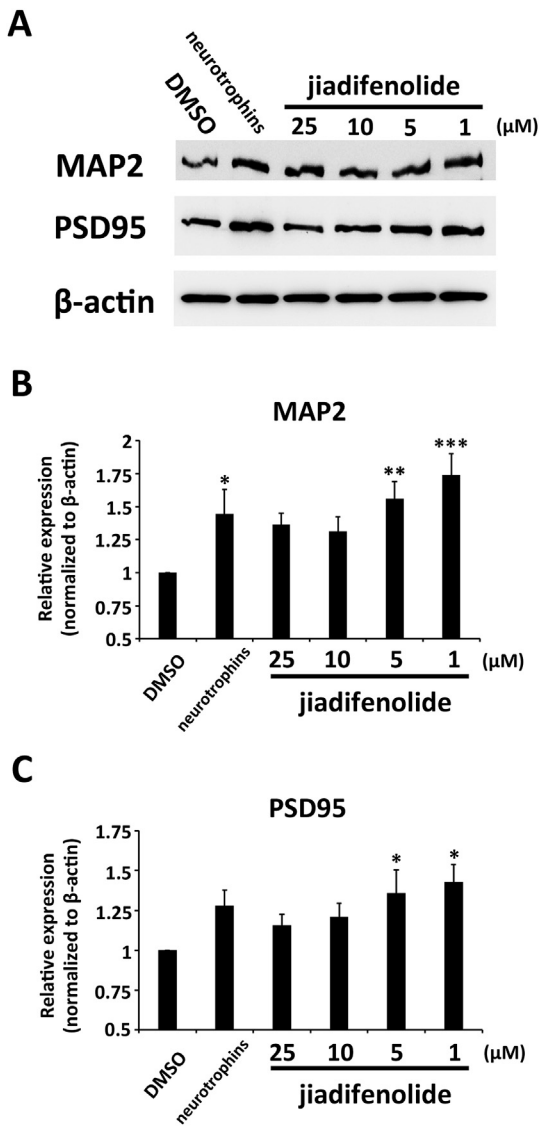

Fig. 4. Western blot analysis of MAP2 and PSD95 expression in jiadifenolide-treated human neuronal cells. At 40 days of incubation (Fig. 2, stage III), cells treated with i) 0.25% DMSO, ii) neurotrophins, or iii) 1–25 μM jiadifenolide were lysed, respectively. Protein expression of MAP2 and PSD95 (a post-synaptic marker) were analyzed in each cell lysate by western blot (A). Band intensities were measured and normalized to that of β-actin. MAP2 (n = 3–4) (B) and PSD95 (n = 4–5) (C) protein levels were normalized to those of DMSO-treated cells (set as 1). Data are presented as the mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for the comparisons of DMSO.

compounds that have a molecular weight (MW) of less than 400 and form eight or fewer hydrogen bonds may cross the blood–brain barrier (BBB) via lipid-mediated diffusion [35]. Jiadifenolide, with a MW of 310.11, has two hydroxy groups in the chemical structure (Fig. 1). Thus, jiadifenolide possesses the properties of a small molecule that is expected to have high BBB permeability. Moreover, Lu et al. recently reported that jiadifenolide can be synthesized in gram amounts using as few as eight steps [36]. Thus, in terms of synthesis efficiency and bioavailability, jiadifenolide is a strong potential drug candidate for clinical-trial evaluation in the treatment of human neurological disorders.

We evaluated the pre-clinical neurotrophic effects of jiadifenolide on human neurons using neuronal cells derived from hiPSCs. By performing image analysis of neuronal cells and MAP2 and PSD95 protein expression, we demonstrated that jiadifenolide promoted dendritic outgrowth, and facilitated cell growth (or prevented death) in human neuronal cells derived from iPSCs. These results suggest that jiadifenolide exhibits neurotrophin-like effects in human neuron, and is a promising drug candidate for treatment of neurological diseases.

Conflict of interest

There are no conflicts of interest to declare.

Acknowledgments

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